

EXHIBIT H

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MINIREVIEW

Identification and Validation of Tumor Suppressor Genes

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Cancers are associated with frequent deletions of genetic material that select for the loss of genes regulating normal cellular physiology. Although several cancer suppressor genes have been identified from these areas of deletion, the identities of the vast majority remain unknown, making approaches leading to their localization, identification, and validation an important continuing endeavor. Those currently characterized cancer suppressors include regulators of aspects of the cell cycle, growth and transcriptional regulators, DNA repair enzymes, differentiation factors, cell motility elements, and regulators of signal transduction. Several inherited cancer predisposition genes have been mapped and cloned using meiotic genetic linkage mapping but less success has been achieved identifying those genes involved in non-familial cancer. The future localization, identification, and validation of these genes are likely to involve a combination of complementary position-oriented and function-driven approaches, some of which are detailed in this article. © 1999 Academic Press

Loss of genetic material from cancer cells occurs more frequently than chromosomal amplifications (1). One consequence of these events is the physical removal or functional inactivation of tumor suppressor genes during the etiology of solid tumors (2). Chromosomal mechanisms that could lead to either increases or decreases in the expression of genes are illustrated in Fig. 1. In this article we will focus on cancer suppressor genes and the various approaches used to localize and identify this category of genes. Loss of function of genes which regulate the normal growth of cells can occur through point mutations or small scale deletions, loss of an entire chromosome (monosomy), chro-

mosomal loss followed by duplication (uniparental disomy), genetic recombination, CpG island methylation or any combination of these mechanisms (2) which are illustrated in Fig. 1. The net result of any of these processes is selection for the loss of function of genes regulating normal cellular physiology thereby leading to an increase in the tumorigenic potential of cells which in turn drives neoplastic progression. Identified cancer suppressor genes include regulators of aspects of the cell cycle, growth and transcriptional regulators, DNA repair enzymes, differentiation factors, cell motility elements, and regulators of cellular signaling (see Table 1).

Microscopic cytogenetic evidence of genetic alterations leading to loss of functional genes has been reported for virtually every cancer and can be evidenced as the deletion of several intact chromosomes and/or subchromosomal regions during tumor development (3). For example, loss of chromosomes X, Y, 4, 10, 13-15, 18 or 22 and/or chromosome segments 1p22-pter, 3p13-pter, 6q14-qter, 8p, 9p, 11p or 17p occur frequently in several common cancers, including carcinomas of the breast, brain, kidney, colon, ovary, lung and skin (1). Several of these deleted chromosomes or subchromosomal regions in tumors have facilitated the isolation of tumor suppressor genes, such as INK4A/p16 in 9p21 (4, 5), RB1 in 13q14 (6) and PTEN in 10q23 (7, 8), and lead to the demonstration that they are functionally inactivated in cancer. Additional cancer suppressors and a list of their chromosomal locations are provided in Table 1.

IDENTIFICATION OF CANCER SUPPRESSOR GENES BY POSITIONAL CLONING

The major successes in mapping and isolating genes that are functionally inactivated or deleted during tumor progression have involved meiotic genetic linkage mapping in familial cancer clusters. Examples illustrating the effectiveness of this approach are the clon-

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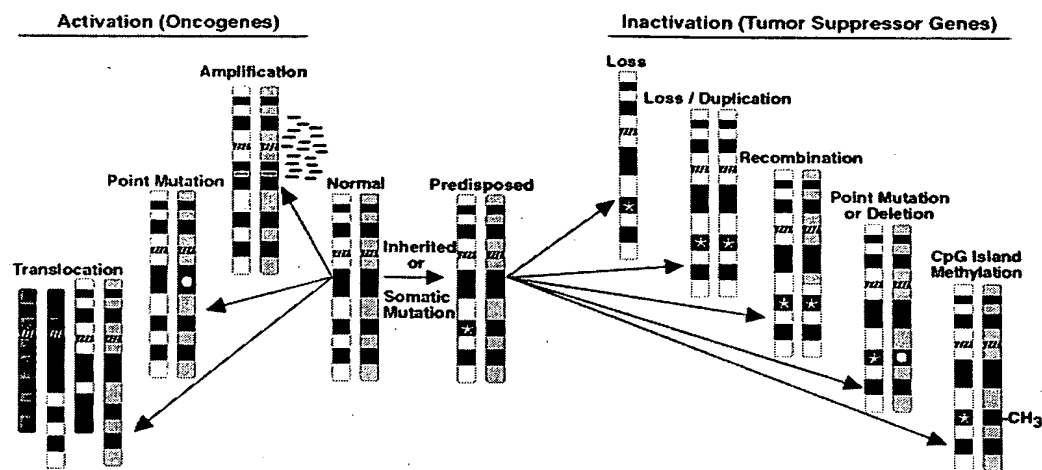


FIG. 1. Chromosomal mechanisms of gene alteration.

ing of the BRCA1 and BRCA2 genes that were linked to genetic predisposition for breast cancer (9–12), the von Hippel-Lindau disease gene (VHL) linked to familial kidney cancer (13) and the APC gene for Familial Adenomatosis Polyposis (FAP) which predisposes affected individuals to colon cancer (14, 15). Since a familial predisposition to cancer accounts for only a small percentage (~10%) of many of today's most prominent cancers, the vast majority arise sporadically and are triggered through a combination of genetic and environmental factors. For example, only ~10% of melanomas (16) and lung cancers (17) occur in familial settings while the remaining ~90% are triggered by environmental stimuli such as severe sun burning during childhood (16) or cigarette smoking respectively (18). Certain familial cancer genes do play significant roles in sporadic cancers and one example is the cyclin dependent kinase inhibitor, INK4A/p16, which is involved in both familial and sporadic melanomas (16). However, the vast majority of cancer suppressor genes significant to the progression of sporadic cancers remain to be identified.

Suppressors with roles in non-familial cancers have been localized and identified through a number of different strategies which are briefly mentioned in Table 1. The most common approach utilizes a combination of cytogenetics and its molecular counterpart, loss of heterozygosity (LOH), to search for increasingly smaller regions of chromosomal loss in tumor cells from which to clone candidate genes by a process termed positional cloning (Fig. 2). Comprehensive cytogenetic analyses performed on collections of tumors have identified non-random areas of loss within particular cancers and common to multiple tumor types (1). In certain instances, the identification of these areas as "hot spots" for loss of genomic material has facilitated the identification of cancer suppressor genes at these sites. For example, the INK4A/p16 gene was identified through

this approach from a region of 9p21 that is commonly deleted during the development of many types of cancer (4, 5). A recent report by Mertens *et al.* (1998) further demonstrates the utility of the cytogenetic survey approach to identify chromosomal regions targeted for losses in several of today's most prominent carcinomas (1). Also, Comparative Genome Hybridization (19) has successfully defined genomic regions of loss in tumor material; a recent example of this strategy has identified several deleted regions in primary melanoma tissue (20). These approaches are complementary and highlight regions lost during tumor progression as possible sites of cancer suppressor gene loss.

Once a chromosomal region has been identified as a target of deletion, the region is narrowed by searching for loss of allelic material in tumors in comparison to normal tissue from the same patient by a process termed LOH. Ideally a small region of homozygous loss can be identified in the tumor material and a physical map spanning the region can be constructed from which candidate genes can be cloned (Fig. 2). Deleted in pancreatic carcinoma, locus 4 (DPC4) was cloned in this manner by identifying a convergent region of homozygous deletion in a number of pancreatic tumor samples (21, 22). Once a region is defined, one approach to identify candidate genes is to examine known cancer suppressor genes or expressed sequence tags (ESTs) that occur in these regions of homozygous or hemizygous deletion to determine if their candidacy can be confirmed by either mutational or functional analysis (see Fig. 2). As more genes and expressed sequences are identified and mapped to subchromosomal regions which are frequently deleted in cancers, determining their involvement in tumor progression will become an increasingly important strategy in the identification of these genes. In many instances only a small portion of sequence need be obtained and then the intact gene can be pieced together using on-line

TABLE 1
Examples of Cloned Tumor Suppressor Genes

Gene	Chromosomal location	Cancer associations	Function	Mode of identification
Cell cycle regulators				
RB1	13q14.2	Retina, lung, bone, bladder, breast, pancreas	Cell cycle regulation	Positional cloning, DNA segment hybridizing to gene
p53	17p13.1	Brain, breast, leukemic cells, (most sarcomas)	Cell cycle regulation, growth arrest, apoptosis	Immunoprecipitates of large tumor antigen
INK4A/p16	9p21	Melanocyte, brain, leukemic cells (ALL), esophagus, lung, bladder, pancreas	Cyclin-dependent kinase inhibitor	Two-hybrid screening
INK4B/p15	9p21			
INK4C/p18	1p32			
WAF1/p21	6p21	Prostate, lung	Cyclin-dependent kinase inhibitor	Anti-cyclin D1 immunoprecipitates
Transcriptional regulators				
E2F1	20q11	Erythroleukemic cells	Transcription factor	Association with pRb
BRCA1	17q21	Breast, ovary	Putative transcription factors	Linkage & positional cloning
BRCA2	13q12-13			
WT1	11p13	Kidney	Transcription factor	Region of heterozygous deletion
VHL	3p25-26	Kidney, central nervous system	Modulates RNA polymerase II via elongin	Linkage & positional cloning
PTC1	9q22.3	Skin (nevroid basal cells)	Transcription repressor	Linkage & positional cloning: sequencing from YAC/cosmid contig
Cell growth regulators				
TGFBR1	9q33-34.1	Colon, retina, liver, stomach	Cell growth inhibitor	Cross-linked to TGF β
TGFBR2	3p21.3-22			
DPC4 (SMAD4)	18q21.1	Pancreas, colon, bladder, biliary	TGF β signaling pathway-cell growth inhibitor	Positional cloning, region of homozygous deletion
DNA repair enzymes				
MSH2	2p22-21	Colon	DNA mismatch repair	Degenerate oligonucleotide PCR
MLH1	3p21.3-23			
Differentiation factors				
DCC	18q21-qter	Colon	Differentiation	Region of heterozygous deletion
Phosphatase				
PTEN	10q23.3	Brain, melanocytes, prostate, thyroid, breast	Phosphatase	Representational difference analysis
Cell motility factors				
CDH1 (e-cadherin)	16q22.1	Breast, ovary, endometrium, liver, skin (squamous cells)	Ca ²⁺ -dependent intercellular adhesion, signaling	Protein isolated from embryonal carcinoma cell membranes
APC	5q21	Colon (FAP)	Binds α - and β -catenin, cell cycle progression	Positional cloning, screening a YAC library with 5q21 markers
MCC	5q21			
Ras regulators				
NF1	17q11.2	Peripheral nervous system, skin	RAS-CTPase-activating protein	Sequencing region of translocation
NF2	22q12	Central nervous system		Linkage analysis, bi-directional cosmid walk

Note. Adapted from Hesketh, R. (1997) The Oncogene and Tumor Suppressor Gene FactsBook, Second ed., Academic Press, London.

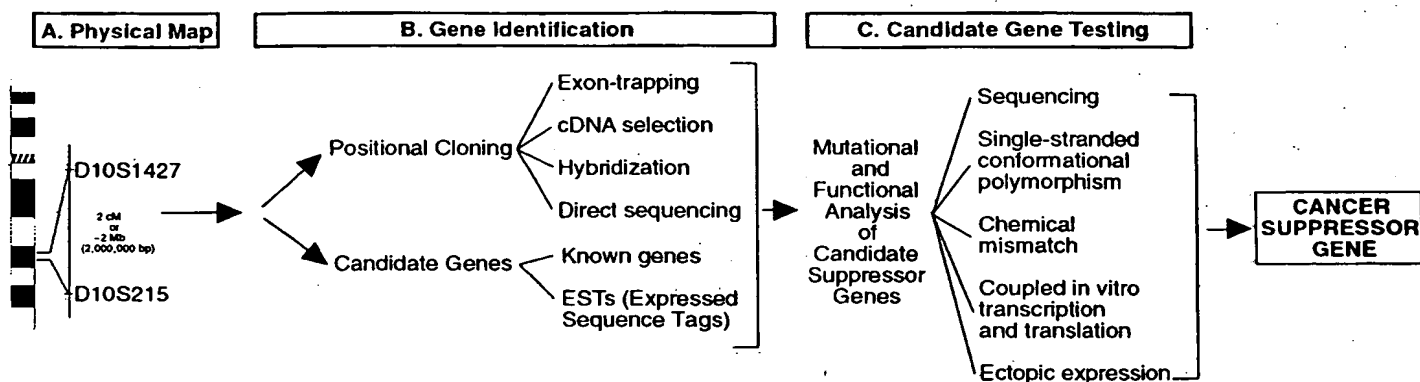


FIG. 2. Position-oriented strategies for gene identification.

data bases such as those of The National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) or The Institute for Genome Research (<http://www.tigr.org/>). A portion of the recently cloned PTEN dual specificity phosphatase suppressor gene was pieced together using this strategy (8). However, if candidate genes can not be identified through this method, the alternative is to directly clone putative suppressors (23, 24) through approaches such as exon trapping, cDNA selection, hybridization strategies or by direct sequencing of the entire genomic region (Fig. 2).

Candidate cancer suppressor genes identified by either approach must be shown to be biologically relevant to the pathogenesis of the cancer being studied (see Fig. 2). Therefore, in addition to the identification of large scale deletions in tumors, further confirmation is provided by the discovery of nonsense and missense mutations which either truncate the protein or affect its normal cellular function in primary tumors and cell lines. Illustrative examples of these types of mutations were identified for the INK4A/p16 (4, 25, 26) and PTEN (7, 27–29) cancer suppressor genes. Approaches such as single stranded conformational polymorphism, chemical mismatch or coupled *in vitro* transcription and translation can aid in the identification of these mutations (23, 24). The ultimate validation is reintroduction of the putative cancer suppressor gene into cancer derived cell lines lacking expression and observing a reversion to a less tumorigenic, more “normal” phenotype. Recent studies showing the effects of ectopic PTEN expression in melanoma and glioma cell lines illustrate the importance of cancer suppressor gene validation (30–33).

Unfortunately, positional cloning approaches are extremely laborious and not always successful. An increasing number of situations have arisen where the cytogenetic and LOH evidence has only been able to define a large region of deletion or where LOH has identified several independent and widely spaced regions of loss within such a region. Obviously, in either circumstance the isolation of the targeted gene is ex-

tremely difficult. For example, a large number of such studies, including several high-resolution LOH screens, have been performed with markers spanning the long arm of chromosome 11 in melanoma (34, 35), breast cancer (36–39), ovarian cancer (40–43), cervical cancer (44, 45), lung cancer (46) and other solid tumors (47, 48). Currently, multiple closely spaced, but non-overlapping, regions of LOH have been detected on 11q, perhaps indicating the presence of several distinct suppressor loci.

Further complications occur in certain cancer types that are associated with a recurring genetic alteration that entails monosomy or uniparental disomy. This, of course, provides little detailed mapping information. Chromosome 10 is an example where tumor-associated monosomy has been reported for several tumor types (1). Localization of the suppressor genes by cytogenetic or molecular strategies alone has been limited largely due to the small proportion of tumor samples which exhibit defining segmental deletions. Clearly, alternative approaches will be required in such cases. One such strategy is to use the growth or tumor reducing abilities of cancer suppressors as a functional approach toward the mapping and identification of these genes. It has been directly demonstrated that functional complementation can localize separate suppressor regions on a single chromosome (33, 49–52), assign varying tumorigenic phenotypes to particular suppressor regions (53–55), and demonstrate the existence of multiple suppressor genes on a single chromosome (55, 56). The remainder of this review will focus on these functional mapping strategies and discuss their utility for the identification of tumor suppressor genes.

FUNCTIONAL MAPPING APPROACHES TO THE IDENTIFICATION AND VALIDATION OF TUMOR SUPPRESSOR GENES

Functional complementation mapping through the introduction of large segments of genomic DNA into

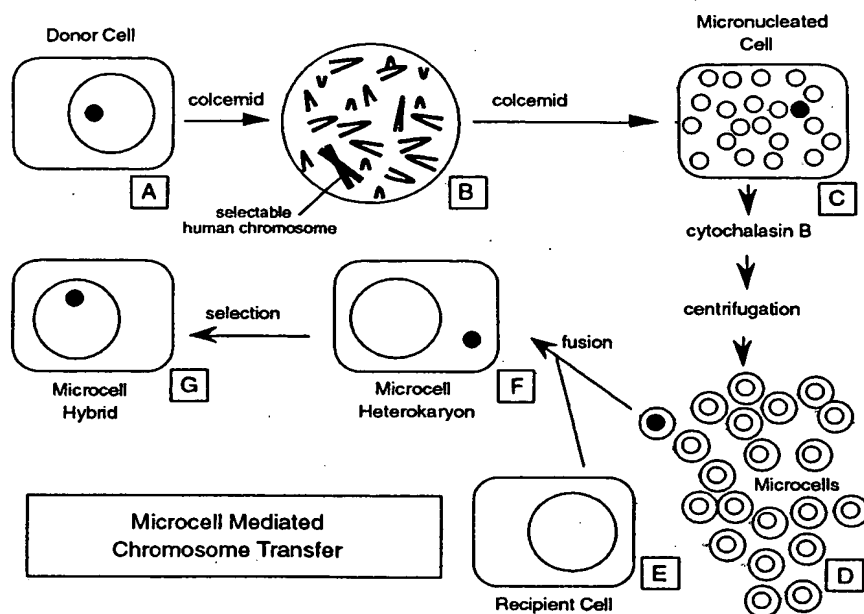


FIG. 3. Diagrammatic representation of the microcell-mediated chromosome transfer technique used to generate somatic cell hybrids. (A, B) A proliferating population of the chromosome donor cell line is treated with $0.02 \mu\text{g/ml}$ colcemid for 48 hours. The donor cell line contains a single copy of a human chromosome that has an integrated dominant selectable marker; for example neo. (C) Colcemid treatment traps the donor cells in mitosis but over time they overcome this block and enter G1. The lack of the mitotic spindle forces the nuclear membrane to re-form around the condensed chromosomes, forming micronuclei. Each micronucleus may contain single or multiple chromosomes. (D) Cytochalasin B (4.85 mg/ml) treatment followed by centrifugation causes the micronucleated cells to enucleate. The micronuclei, surrounded with a thin layer of cytoplasm and cell membrane are referred to as microcells. These micronuclei are filtered to enrich for those containing single chromosomes. (D, E) The microcells are then fused using polyethylene glycol to the recipient cancer cell lines. (F, G) Only potential hybrids containing the transferred human chromosomes with the integrated selectable marker are capable of surviving in selective medium.

cancer cells can demonstrate the presence of cancer suppressing activity and delineate chromosomal regions as the sites for the responsible genes. Mapping in this manner proceeds progressively from an intact chromosome, to a subchromosomal fragment, to a yeast artificial chromosome (YAC), to a bacterial artificial chromosome (BAC/PAC/P1), and finally to the identification of the gene itself. Since the process is accompanied by a scorable phenotype, once the gene is identified its validation can be confirmed if ectopic expression of the gene results in similar phenotypic effects.

Initially, potentially important chromosomes or subchromosomal regions are identified as possible sites of suppressor gene loss through a combination of both cytogenetic and LOH studies. A normal wild-type counterpart to this chromosome that is tagged with an integrated dominantly selectable marker, is then transferred into tumor-derived cell lines. The process by which a normal chromosome is moved into a cancer cell line is termed microcell-mediated chromosome transfer (57) and is briefly described in Fig. 3. It is the most efficient and reliable means of transferring specific, intact chromosomes or subchromosomal fragments into cultured cells, and donor cell lines are now

available for the transfer of most of the human chromosome complement. Panels of rodent donor cell lines which contain single human chromosomes can be purchased from sources such as the American Tissue Culture Collection (<http://www.attc.org>) or the Coriell Cell Repositories (<http://locus.umdj.edu/nigms/>). In most cases, the human chromosome contains an integrated dominant selectable marker, such as neomycin phosphotransferase (neo), thereby forcing its retention and allowing its transfer and selection in human cell lines.

Following transfer, hybrid cell lines are both characterized to determine whether they contain only the transferred human chromosome and phenotyped to determine the effect that the transferred chromosome has on the cancer cells. It is important to show that only the single transferred human chromosome is present in these hybrids since whole cell hybrids, created by the fusion of both donor and recipient cell lines, often exhibit complicated confusing phenotypes due to the presence of portions or the entire rodent genome in these cells. Chromosomes transferred by this technique can be identified and studied by molecular strategies, conventional chromosome stains or by fluorescence *in situ* hybridization in order to confirm transfer of intact stable chromosomes (49, 50, 54).

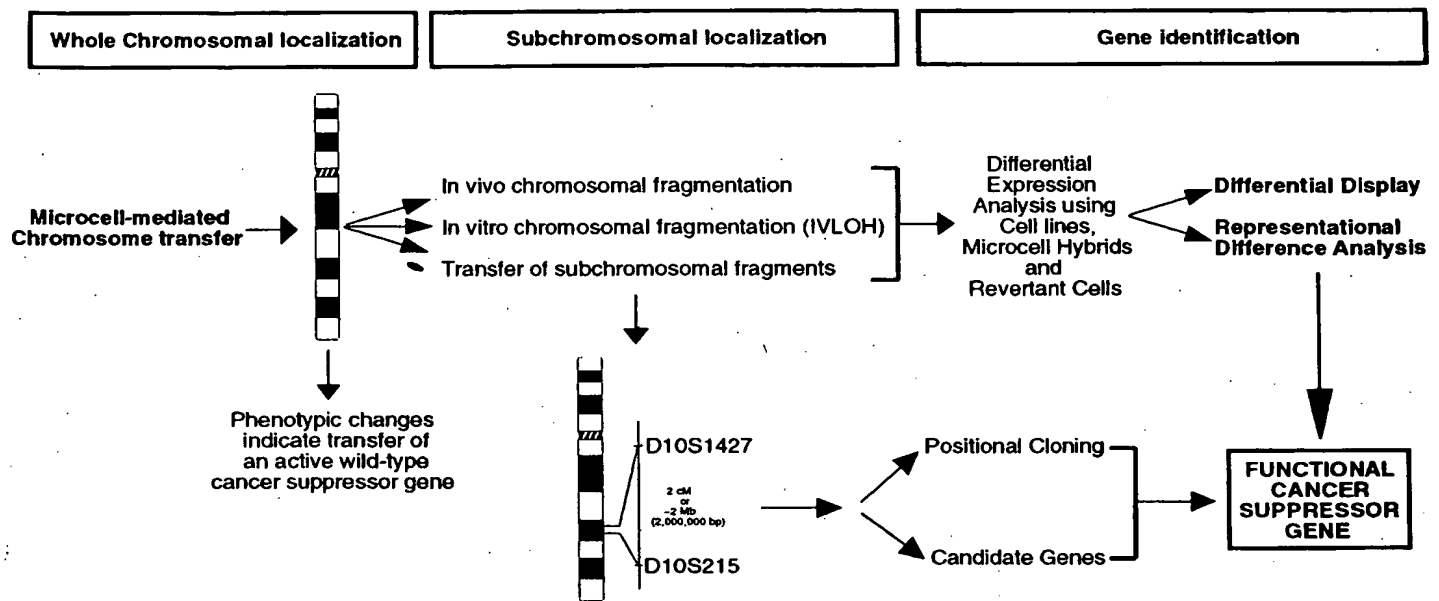


FIG. 4. Functional mapping of cancer suppressor genes.

Clonal hybrid cell lines containing only the transferred chromosome are then tested for effects resulting from the reintroduction of putative tumor suppressor genes that were lost during tumorigenesis (Fig. 4). Reduced growth rate, more normal morphological appearance, decreased ability to form colonies in soft agar, reduced metastatic potential, lack of angiogenic capacity or a reduced ability to form tumors in nude mice all lead to the conclusion that an active tumor suppressor gene was reintroduced with the transferred chromosome (49–52, 58). An added advantage of this system is that the activity of genes on the transferred chromosome can be studied in the recipient cells with less concern for several potential artifacts that can arise with other gene transfer methods. These cancer suppressor genes are expressed at normal physiological levels since they remain coupled to their own cis-regulatory regions and are associated with chromatin that more closely resembles the native structure. Therefore the biological effects and measurable phenotypes are not due to drastic overexpression which can be observed when using ectopic expression constructs.

Additional confirmation that the introduced chromosome is responsible for the phenotype is obtained when hybrid cells which formed tumors in nude mice select against the transferred chromosome and segregate it thereby reverting to a tumorigenic phenotype (49–52). However, whole chromosome segregation during tumor formation does not always occur, and several studies have reported loss of smaller chromosomal regions which can then be used to infer the location of the genes responsible for the effect (Fig. 4). Molecular examination of tumorigenic revertants to search for loss

of a particular portion of the transferred chromosome was used by Misra *et al.* (1989) to localize a tumor suppressor active in HeLa cells to 11q13-23 (59). While this approach has been used to localize a number of important suppressor sites critical to several cancers (60–62), a major limitation is that there is no control over the size of the chromosomal region(s) lost and they may remain extremely large; thereby not aiding significantly in the mapping effort.

Fragmentation of a transferred chromosome can also occur in culture if the effects of introduced genes have negative consequences for growth of the cells (Fig. 4). This process, which we have termed *in vitro* loss of heterozygosity (IVLOH) (33), can define the site and lead to the identification of the putative suppressor gene; this is shown diagrammatically in Fig. 5. Through this process a normal copy of chromosome 10 was transferred into melanoma cells which then eliminated the responsible growth suppressor gene(s) in culture through chromosomal fragmentation accompanied by loss of a region extending distally from 10q23.1 (33). Breakage could be detected in every chromosomal band from the centromere to 10q23. Since the PTEN tumor suppressor mapped to 10q23, it seemed reasonable to propose that it might be triggering the chromosomal fragmentation; this was subsequently confirmed (33). The cell line used in this system transcribes a mutant PTEN mRNA but did not produce functional protein because both copies contained an identical T to G truncating point mutation at codon 76. Additionally, subsequent ectopic gene expression studies, in this melanoma cell line which lacked PTEN protein, confirmed the candidacy of PTEN as the driving factor for IVLOH (33).

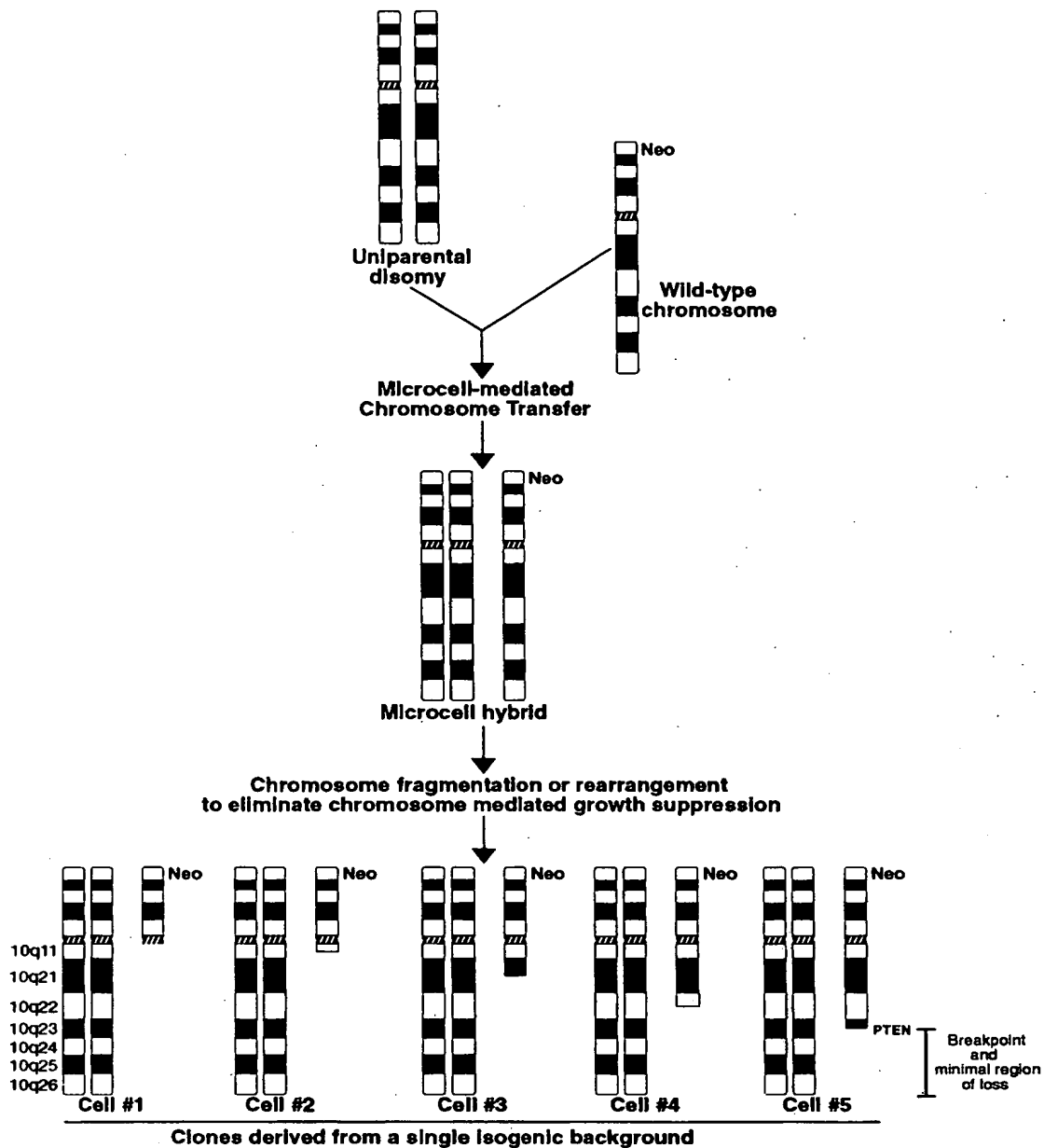


FIG. 5. Functional mapping by *in vitro* loss of heterozygosity (IVLOH).

The genetic content of the cell line to be used for IVLOH complementation is critical to the success of functional mapping. For example, the melanoma cell line UACC 903 was ideal for chromosome 10 complementation since during its evolution it had lost one copy of chromosome 10 and duplicated the remaining one resulting in uniparental disomy (33). Under the circumstance of two identical chromosomal copies, any mutations or deletions would be duplicated and fit the two-hit-model for tumor suppressor genes (2, 63). Therefore cell lines to be used for IVLOH should be

examined prior to transfer to determine that they fulfill these requirements. The reintroduction of a wild-type chromosome 10 into the UACC 903 melanoma cell line restored PTEN suppressor gene activity, disrupting the homeostasis of the cell line and cause both *in vitro* IVLOH and an *in vivo* reduction in tumorigenicity to attenuate the chromosomal-mediated growth control (33).

Another strategy that has been employed to provide a more refined, subchromosomal localization of cancer suppressor genes is to use microcell-mediated transfer

of specific subchromosomal fragments into cancer cells (Fig. 4). Like intact chromosomes, chromosome fragments are "tagged" with an integrated selectable marker which is used to facilitate transfer. The location of the integrated selectable marker within the fragment is important since transferred fragments tend to preferentially contain portions of the chromosome surrounding the site of integration.

Two general approaches have been used to transfer chromosome fragments into tumor cells. The first approach, developed by Koi *et al.* (1993), compartmentalized the transferable human chromosome into microcells and then used gamma radiation to fragment the chromosome (64). This compartmentalization served to minimize the complexity of the fragments contained in these hybrids by separating them from most of the rodent chromosomes at the time radiation was applied and decreased the potential for inter-chromosomal rearrangements involving mouse chromosomes. In any case, small fragments must be translocated onto host chromosomes in order to be stably maintained. A disadvantage of this approach is that extensive hybrid characterization was required after transfer to determine which chromosomal fragments were present in the hybrid cells.

A second strategy which does not require an extensive post-transfer characterization of hybrid cells utilizes panels of rodent cell lines which contain single transferable chromosomal fragments (53, 65). These donors were extensively characterized in a rodent background prior to transfer into cancer cells and consist of homogeneous cell populations containing a single, distinct fragment that is stably maintained in culture. Following transfer into cancer cells the resultant hybrids then require less characterization and only confirmation that the fragment was successfully transferred (53–55). We have generated, characterized and demonstrated the suppressive effects of genes contained within fragments derived from human chromosomes 10 and 11 (54, 55). An added advantage of this approach is that the fragments themselves can break during the transfer process thereby generating stable hybrids containing varying portions of the original fragment and thus limit the number of transfers that are required (54). In these situations, comparisons between suppressed and non suppressed hybrids containing variable portions of the transferred fragment can be used to successfully localize cancer suppressor genes. This approach can also unmask suppressors that have weaker effects and that are usually masked by stronger ones making their localization impossible until the dominant suppressor is eliminated by deletion or mutation (55, 56). A recent report by Parris *et al.* (1998) has shown that this situation occurs on 9p where INK4A/p16 masks the effects of another nearby suppressor (56). Transfer of a chromosome lacking this region reveals the presence of the second functional

suppressor gene. Once a functional suppressor region has been identified, positional cloning and candidate gene testing can be used to examine the candidacy of putative cancer suppressor genes (Fig. 4).

Parental cell lines, derivative microcell hybrids and their revertant counterparts have been used to identify candidate cancer suppressor genes through subtractive expression approaches, such as differential display (66) (Fig. 4). Several investigators have recently reported the identification of differentially expressed candidate genes in microcell hybrids, matched revertant cells and the parental cell population (67–69). These differentially expressed genes map to both the transferred chromosome and to other chromosomes; it is possible that these genes play roles in related signal transduction cascades resulting in tumor suppression. This approach may lead to the cloning of many candidate suppressor genes in the future.

Functional localization using yeast (YACs) and bacterial (BACs/PACs/PIs) artificial chromosomes can further functionally narrow the sites of suppressor genes. Successful expression of YAC-encoded genes transferred into mammalian cells can be achieved in situations where the location of the gene is already known to a fairly high degree of resolution (70, 71); thus vectors can be chosen with a high likelihood that they will contain a complete and functional copy of the gene. Additionally, recent studies by Koreth *et al.* (1999) and Murakami *et al.* (1998) showed that complementation mapping using YACs can localize tumor suppressor activity to a region previously implicated by chromosome transfer and LOH (72, 73). However, the transfection of such large cloning vectors directly into cultured mammalian cells continues to be hindered by substantial technical limitations; the most important of these being the relatively low efficiency with which stable transfectants can be recovered. This severely constrains the number of constructs that can be tested in a reasonable number of transfers. Also, in many cases yeast or bacterial artificial chromosomes require "retrofitting" with a selectable marker or DNA replication origin appropriate for mammalian cells (74, 75). However, BACs are now available from the Roswell Park Cancer Institute (<http://bacpac.med.buffalo.edu/>) that contain the dominant selectable markers blastidicin-s-methylase allowing their transfer without prior retrofitting; this type of vector could increase the utility of BACs for functional complementation mapping. Once a BAC with a suppressive phenotype has been identified, candidate genes can be isolated by either sequencing the entire insert or by approaches such as exon trapping.

CONCLUDING REMARKS

Both positional cloning and functional mapping can successfully move a gene mapping project from the

cytogenetic to the molecular scale. Therefore the localization, identification and validation of these genes in future years will likely involve a combination of complementary position-oriented and function-driven approaches; the recently cloned PTEN tumor suppressor was identified using this strategy (8). Suppressor function was initially mapped to chromosome 10 (76), then sublocalized on 10q (77), and finally the PTEN gene was cloned from a subchromosomal region containing tumor suppressing abilities in microcell hybrids and deleted in primary tumor material (8). This combinatorial approach could be the first of many demonstrations leading to the localization and identification of non-familial cancer suppressor genes.

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